Impact of non-small-cell lung cancer-not otherwise specified immunophenotyping on treatment outcome.

IMPACT OF NON-SMALL CELL LUNG CANCER-NOT OTHERWISE SPECIFIED IMMUNOPHENOTYPING ON TREATMENT OUTCOME

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ABSTRACT

Introduction. The vast majority of Non-Small Cell Lung Cancers (NSCLCs) presents as advanced disease and histological diagnosis is widely based on small samples. The differential activity and toxicity profile of new cytotoxic and molecular targeted therapies according to histotypes requires a precise subtyping of NSCLC. Immunohistochemistry (IHC) contributes to define the most probable histotype, however, the real impact of IHC characterization of NSCLC-Not Otherwise Specified (NOS) in terms of outcome is not well established. **Methods.** A large series of 224 advanced "*non-squamous*" NSCLC diagnosed on small biopsy or cytological samples and homogeneously treated was retrospectively selected, all having adequate follow-up data available. Reviewed diagnoses resulting into two groups: adenocarcinoma (ADC) and NSCLC-NOS. The latter was further characterized by IHC (TTF-1, Napsin-A, p40 and Desmocollin-3) to identify a possible, most probable differentiation lineage. **Results.** 67% of cases were classified as ADC based on morphological examination only ("morphological ADC") and 33% NSCLC-NOS. IHC profiling of NSCLC-NOS identified 43.2% of cases with an ADC immunophenotype ("NSCLC favor ADC"), 10.8% with a phenotype favoring squamous lineage and 46% lacking differentiation features. Survival curves confirmed no difference in terms of outcome between the "morphological ADC" and the "NSCLC favor ADC" groups, while a significantly poorer outcome was found in the "null" group either in terms of best response, progression free and overall survival. **Conclusion.** Tumors with an IHC profile ADC-like had an overall survival comparable to that of "morphological ADCs". These findings support the use of IHC to optimize lung cancer histological typing and therapy.

INTRODUCTION

The majority of Non-Small Cell Lung Cancers (NSCLC) present at advanced stage and the histological definition is widely based on small biopsy or cytological samples. The differential activity and toxicity profile of new cytotoxic agents and molecular targeted therapies according to different lung cancer histotypes[1, 2] led to an increased need for a precise NSCLC subtyping[3] and the differentiation between adenocarcinoma (ADC) and squamous carcinoma (SQC) is the minimum requirement. Unfortunately, in most cases there are only limited amounts of tumor tissue obtained from primary or metastatic sites, generally through fine needle aspiration cytology or tiny bronchoscopic biopsies, available for pathological examination. This may hamper the precise tumor definition, either because of scant viable cells or poor tumor differentiation[4]. In such a context, morphological diagnostic criteria could fail, particularly in undifferentiated cancers. The ATS/ERS/IASLC guidelines recommend the use of immunohistochemistry (IHC) in biopsy samples when a precise morphology-based subtyping is not possible[5]. As a consequence, several studies proposed different panels of IHC markers, useful to identify the specific cell lineages. These IHC markers helped to distinguish SQC from ADC, not only in surgical material[6-8], but also in cytology[9] or biopsy samples[10-13]. Recently, our group demonstrated that a limited, four-marker panel (TTF1, p63, Desmocollin-3 and Napsin-A) could narrow the percentage of unclassified NSCLC-NOS from 36% to 14%, thus contributing to refine lung cancer classification in fine needle aspiration biopsies[14].

However, the real impact on the patients' outcome of IHC-based subtyping of morphologically undifferentiated NSCLCs-Not Otherwise Specified (NOS), compared to the behavior of cases having morphology-driven diagnoses, has not been established. In the present study, we retrospectively analyzed a consecutive series of patients with advanced NSCLC and a *non-squamous* histological diagnosis (ADC and NSCLC-NOS), candidate for first line treatment, for whom small biopsies or cytology specimens only were available. The group of lung cancers

subtyped by an IHC marker panel was correlated with two separate groups of morphology-only ADC and of NSCLC having a "null" phenotype (according to the markers used here), with respect to the response to treatment and outcome.

MATERIALS AND METHODS

***Case selection*.** A cohort of 224 consecutive patients with advanced NSCLC (IIIB and IV stages, UICC TNM 6th edition) diagnosed as *non-squamous* NSCLC (ADC or NSCLC-NOS) on small biopsy or cytological samples and treated at the Thoracic Oncology Unit of San Luigi Hospital (Orbassano-Turin, Italy) from 2005 to 2010 was retrospectively selected. All considered specimens were obtained from chemo-naive patients, who subsequently received first-line treatment; data on response and overall survival were available for all considered patients. Almost all patients received front-line platinum-based chemotherapy with/without experimental agents. Twelve patients with PS2 received single agent pemetrexed or erlotinib/gefitinib (n=2). Forty–one patients were treated second-line (n=22)/third line (n=19) erlotinib according to the registration label. For institutional policy in that period of time, patients with ADC or any other type of NSCLC were not routinely checked for Epidermal growth factor (EGFR) mutation or ALK translocation.

All pathological diagnoses were reviewed (LR) and segregated into two groups: 1. ADC based on morphology only and 2. NSCLC-NOS. An external unrelated pathologist (GR) reviewed all the cases of this latter group confirming that they were all undifferentiated cases with no morphological criteria helpful to discriminate between adeno- or squamous differentiation. Furthermore, those cases with cytological characteristics suggestive of neuroendocrine differentiation (large cell with homogeneous salt-and-pepper chromatin appearance, large nucleoli, abundant granular cytoplasm) were excluded from the series and in those doubtful cases immunohistochemistry for neuroendocrine markers was performed to further exclude positive cases. The NSCLC-NOS group

was further analyzed for a tissue sparing, minimalist IHC approach (as previously described[14]) to better characterize any residual differentiation lineage.

Immunohistochemistry. Five micron-thick serial sections were collected onto charged slides, dewaxed, rehydrated in pH 7.5 buffer, and processed for standard IHC staining. Briefly, after blocking endogenous peroxidase activity in 0.3% hydrogen peroxide and methanol solution for 15 min, 5 micron-thick cell block sections were reacted for 40 minutes at room temperature with the nuclear markers TTF1 (MoAb clone 8G7, 1/100) and p40 (MoAb clone BC28, prediluted) in a first run and with the cytoplasmic marker Napsin A (MoAb clone TMU-Ad02, 1/100) and with the cell membrane desmosomal marker Desmocollin-3 (DSC3, MoAb clone DSC3, 1/30, overnight at 4°C). Slides were then incubated in a detection kit (EnVision Plus HRP, Dako) according to the manufacturer's instructions, developing peroxidase activity with 3-3'-diaminobenzidine. Antigen retrieval was performed in a pressure cooker for five minutes at 125°C followed by a quick 10 second step at 90°C using pH 8.0 EDTA buffer for all primary antibodies, and pH 6.6 citrate buffer for DSC-3. Finally, slides were counterstained with hematoxylin, dehydrated and mounted. The specificity of all immunoreactions was double-checked by substituting the primary antibody with a non-related isotypic mouse immunoglobulin at a comparable dilution, and with normal serum alone. All histological bioptical or cytological cell blocks were used for immunohistochemical reactions. Normal bronchial epithelium and alveolar epithelium were used as internal controls for basal and glandular markers, respectively. TTF1 and p40 were considered positive when a nuclear signal of any intensity was recorded; Napsin A was considered positive when a finely granular intracytoplasmic signal was found; DSC3 was considered positive in case of weak linear membrane signal.

Statistical analyses. Qualitative data were compared by Fisher's *t* test. Overall survival (OS) was defined as the time between the date of diagnosis and the last follow-up and/or death, and progression free survival (PFS) was calculated from the date of diagnosis to the date of clinical

and/or radiological progression to the first line treatment. Best response to therapy was recorded as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD), following RECIST criteria[15,16]. Disease Control Rate (DCR) and Response Rate (RR) percentages were calculated on the basis of best responses. Survival estimates were calculated using the Kaplan-Meier's method and compared by the log rank test. Cox's univariate survival analysis was performed to identify prognostic factors for both Progression Free Survival (PFS) and overall survival (OS). All analyses were performed using the GraphPad Prism statistical software. All *P* values were based on 2-sided test and considered as significant when less than 0.05, confidence intervals (CIs) at the 95% level.

RESULTS

Immunohistochemical subtyping. Patients' characteristics are summarized in **Table 1.** After review, diagnoses were distributed as follows: 150/224 (67%) were ADC based on morphological examination only, while the other 74/224 (33%) were NSCLC-NOS (**Figure 1)**. After applying a panel of four markers (TTF1, p40, DSC3 and NapsinA) the NSCLC-NOS group was further divided as follows: 32/74 (43.2%) cases resulted TTF1 and/or Napsin A positive (and p40/DSC3 negative), and were subtyped as "NSCLC favor-ADC" based on a glandular immunophenotype; on the other hand, 8/74 (11%) cases resulted p40 and/or DSC3 positive (and TTF1/Napsin A negative), being subtyped as "NSCLC favor SQC" according to a squamous phenotype. This group was subsequently excluded from the statistical analyses due to the small number of cases and because this study was designed for "*non-squamous*" NSCLC to better understand the impact of diagnostic workup on therapeutic decision. Finally, 34/74 (46%) cases did not reveal any specific immunoprofile and were consequently classified as "NSCLC with a null phenotype" (including nine cases that remained "not otherwise specified" due to insufficient material for IHC). No significant correlations were found among groups and clinical-pathological characteristics.

Response to therapy and survival. Response rate (RR) and disease control rate (DCR) percentages were calculated for each group (**Figure 2**). In terms of response to therapy, the group of patients with a diagnosis of morphological ADC had 46 PR, 54 SD and 50 PD with a RR=31% and DCR=67%. The whole group of morphologically NSCLC-NOS had 28 PR, 18 SD and 28 PD with a RR=38% and DCR=62% with no significant difference between the two groups. However, after IHC, the subgroup of patients with a diagnosis of "NSCLC favor ADC" had 14 PR, 10 SD and 8 PD with a RR=44% and a DCR=75%, a finding not significantly different from the group of "morphological ADC". Conversely, the subgroup of NSCLC with a null immunophenotype had 12 PR, 5 SD and 17 PD corresponding to a RR=35% and DCR=50% all significantly different either with the "morphological ADC" cohort (Chi-Square test, p=0.009) or the "NSCLC favor ADC" subgroup (Chi-Square p=0.01), or even the whole series of ADC differentiated tumors (with morphological or immunophenotypical ADC features) (Chi-Square test, p=0.006).

In terms of outcome, the "morphological ADC" had a median PFS of 7.3 months, a median OS of 12.3 months with 10/150 (6.7%) censored cases. On the other hand, the group of NSCLC-NOS had a median PFS of 5.9 months (HR= 0.6249; 95% CI=0.4571 to 0.8544, p=0.003) and a median OS of 9.6 months with only 1/74 (1.3%) censored case (HR= 0.5804; 95% CI= 0.4214 to 0.7995, p=0.0009) (**Figure 3**). After IHC, the "NSCLC-favor-ADC" cases had a median PFS of 7.05 months, a median OS of 13.1 months and 1/32 (3.1%) censored case, while the group of "NSCLC-null phenotype" had a median PFS of 3.8 months (log-rank test p<0.0001) and a median OS of 7.2 months without censored cases (log rank test p<0.0001) (**Figure 4A**). Grouping together all the ADC differentiated tumors, a significant difference compared to the "NSCLC null phenotype" group was confirmed both in terms of PFS (HR= 0.2193; CI= 0.1149 to 0.4184, p<0.0001) and of OS (HR= 0.2819; CI= 0.1664 to 0.4773, p<0.0001) (**Figure 4B**).

In the "morphological" ADC subgroup, 20% of the patients received Erlotinib as second/third line, while the same agent was part of second/third line treatment in 22% of the NSCLC-NOS favor ADC and 12% of the NSCLC "null" subgroups.

DISCUSSION

Data about the benefit of immunohistochemical subtyping in terms of disease outcome are still unclear[17], even though there is the almost mandatory need for accurate histotyping of NSCLC because of the demonstrated efficacy of histology-driven treatment decision. In this study, we show that immunophenotyping of (poorly differentiated) NSCLC is a useful task in terms of therapeutic strategy. In fact, cases with an undefined morphology pattern but an immunophenotype that overlaps that of well differentiated adenocarcinoma ("NSCLC favor ADC" subgroup) have both response to chemotherapy and outcome similar to that of "morphology-only" ADC cases. In addition, in terms of response rate and survival, such treatment outcome was significantly different from the NSCLCs subgroup having a "null" phenotype. The latter followed a more aggressive course, possibly as the result of an undifferentiated nature of this subgroup of tumors.

This study was restricted to patients with advanced NSCLC that underwent chemotherapy in a period of time (2005-2010) prior to European Medicine Agency (EMA) approval of pemetrexed (PEM) as first line treatment for advanced *non-squamous*-NSCLC and whose *non-squamous* carcinoma diagnoses were obtained on small biopsies or cytological samples. On the one side, a strength of this study is the homogeneous patient sample in terms of disease stage and pathological diagnoses, while one potential weakness may be its heterogeneity in terms of chemotherapy treatment options. However, this turned out to be irrelevant because the observed results (rather related to diagnostic workup and its impact on outcome), were independent from a specific chemotherapy regimen. Out of the initial cohort of 224 consecutive carcinomas, 74/224 (33%) could not be characterized on the basis of morphological features alone, and remained NSCLC-

NOS. These two groups did not differ in terms of best response to therapy but only in terms of PFS or OS. It could be argued that the use of EGFR–TKI, in front or subsequent lines may be of influence in improving PFS and OS data. It should be noted that only 2 PS2 patients received front line EGFR-TKI with an unknown EGFR mutational status while the percentage of patients in both "morphological" ADC and NSCLC-NOS treated with second/third line EGFR-TKI was quite similar (20% versus 16%) making unlikely the influence of any potential bias in PFS and OS values. In addition is of note that PFS and OS values reported for "morphological" ADC and IHC "favor ADC" subgroups are quite similar to those reported in more recent phase III clinical trials testing chemotherapy plus/minus experimental agents in all comers[18,19].

Immunohistochemistry allowed to further stratify such apparently undefined tumors, identifying 32/74 (43%) cases sharing glandular markers. Such cases had a rate of response to chemotherapy highly similar to that of morphology only ADC diagnoses (Chi-square test, p=0.3), as well as a strongly similar trend in terms of PFS (Mantel-Cox test, p=0.2) and OS (Mantel-Cox test, p=0.3), thus confirming their relationship with a glandular differentiated lineage. A small subgroup (9/74 cases, 12%) showed a squamous carcinoma phenotype after IHC analyses: these cases were not further considered, either because of the small size of the group and for being out of the specific aim of the study. The remaining cases had a "null" phenotype (25/74 cases, 34% of NSCLC-NOS and 11.2% of the whole cohort) that included also nine cases with insufficient material in the block to perform IHC and coded as NSCLC-NOS, without impairing the statistical analyses. The survival outcome of this group, was significantly worse than those of the above subgroups ($p<0.0001$), although comparable in terms of disease stage and treatment. The response rate (RR) was determined by the number of partial responses (PR) recorded over the total amount of cases in this group. This value (35%) was quite similar to the value of morphological ADC (31%) probably due to some partial responses to therapy recorded in the NSCLC null group in a limited interval time of the disease that was not maintained in the overall survival: as a matter of fact the difference

between the two groups was greater in term of DCR with a major number of progressive disease cases in the undifferentiated group. In NSCLC-NOS the "null phenotype" appears to be an adverse prognostic factor on treatment outcome, independently from the type of chemotherapy. These findings are not surprising since it is known that large cell carcinomas, according to the WHO terminology for surgically resected undifferentiated lung cancers or more in general undifferentiated lung carcinomas (i.e sarcomatoid carcinomas) usually follow a more aggressive course[20].

Nevertheless, it is of clinical interest that undifferentiated carcinomas may be accurately characterized using a limited panel of IHC markers in small amounts of tumor tissue. Usually, when standard molecular diagnostic procedures are used, these undifferentiated carcinomas do not harbor EGFR and ALK abnormalities at a rate as ADC does[21] and these tumors are only candidates for cytotoxic therapy: the use of a cheap additional diagnostic work-up through IHC may allow a better treatment customization in today practice, since new drugs with differential activity according to the histological type are available. In addition, the identification of a NSCLC-NOS "null" subgroup may represent an opportunity for a more in depth assessment of its specific molecular profile. A recently published paper that incorporated in the classification of undifferentiated large cell carcinoma the next-generation sequencing technology together with an IHC algorithm, not only confirmed several of the IHC assignments but afforded classification in cases where definite pathological diagnosis was not possible[22]. While the value of this approach may become relevant in the near future to detect specific gene alterations, it still needs further validation. The technology is not available everywhere while the information provided by IHC are readily affordable in routine clinical practice.

An additional value of this report is the possibility of optimally stratifying NSCLC cases even using small biopsy samples. The difficulty of defining the subtype based on morphology alone, could depend either on the intrinsic features of the tumors or on the amount of diagnostic cells. In this

study, 58% of cases were cytological samples, a percentage that rose up to 76% when only the NSCLC-"null" category was considered. When processing such samples, it has to be kept in mind that the recommended call for a precise immunophenotyping[23] has to be well balanced with the parallel recommended call for preserving tissue for further molecular analyses[24,25]. In fact, an accurate subtyping may require, in selected cases, the investigation of multiple markers with the consequence of using most if not all the available tissue. Eventually, this will provide a pathological characterization of the tumor, to the cost of the possible loss of further chances of molecular characterization. A possible solution is to rigorously select the panel of IHC markers when tiny biopsies or extremely undifferentiated tumors are analyzed, which are expected to require many more markers. In our[14] and others[24] experience, the first choice differential markers for glandular and squamous lineages should include nuclear markers, which are more easily assessed in small specimens, even if suffering from sampling artifacts or poor cellularity due to necrosis. TTF-1 and p40 are currently the best options to differentiate adenocarcinoma from squamous carcinoma histotypes, respectively. In particular, TTF-1 performs better using 8G7 monoclonal antibody, in consideration of its higher specificity[26], and p40 is superior to p63 again in consideration of its higher specificity[27,28]. When both markers turn out to be negative, in approximately 15-20% of cases[26], it is worth testing a second set of markers before concluding for a diagnosis of "null phenotype". To this purpose, rather than relying on specific cytokeratin (CK) types (e.g. CK 7 or CK 5&6)[29,30], we preferred to rely on other lineage markers, that in our[31] and others'[32,33] experience were associated to a higher specificity. These included Napsin A for adenocarcinoma lineage and the desmosomal marker DSC3 for squamous differentiation, which allowed to refine the final diagnosis in an additional group of tumors[34].

The apparently high rate of NSCLC that retain a "null" phenotype after IHC does not contrast with published data[35] in which the rate of unclassified NSCLC-NOS is reported to be inferior to 10%, especially when surgical series only were investigated. In the current series, a small percentage of

cases (12%) resulted to be not further characterized for the insufficient number of diagnostic cells available for IHC investigations. Despite the official WHO 2004 classification relies on the exclusive role of morphology, the combined approach proposed in this retrospective study is totally aligned with the recently proposed diagnostic algorithms for NSCLC histotyping in small biopsy or cytology samples[36].

In conclusion, this study indicates that at least in the group of lung adenocarcinomas, those cases categorized as "NSCLC- favor ADC" have a similar clinical behavior as conventionally diagnosed ADC. This further supports the validity of a major effort in accurately subtyping all apparently undifferentiated lung carcinomas, using a combined morphological and immunophenotypic approach in all small biopsy or cytology samples from advanced NSCLC patients.

REFERENCES

1. Neal JW. Histology matters: individualizing treatment in non-small cell lung cancer. *Oncologist* 2010;15:3-5.

2. Stinchcombe TE, Grilley-Olson JE, Socinski MA. If histology matters. *J Clin Oncol* 2010;28:1810-1812.

3. Rossi G, Pelosi G, Graziano P, et al. A reevaluation of the clinical significance of histological subtyping of non--small-cell lung carcinoma: diagnostic algorithms in the era of personalized treatments. *Int J Surg Pathol* 2009;17:206-218.

4. West H, Harpole D, Travis W. Histologic considerations for individualized systemic therapy approaches for the management of non-small cell lung cancer. *Chest* 2009;136:1112-1118.

5. Travis WD, Brambilla E, Noguchi M, et al. Diagnosis of lung cancer in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung

Cancer/American Thoracic Society/European Respiratory Society classification. *Arch Pathol Lab Med* 2013;137:668-684.

6. Kargi A, Gurel D, Tuna B. The diagnostic value of TTF-1, CK 5/6, and p63 immunostaining in classification of lung carcinomas. *Appl Immunohistochem Mol Morphol* 2007;15:415-420.

7. Jagirdar J. Application of immunohistochemistry to the diagnosis of primary and metastatic carcinoma to the lung. *Arch Pathol Lab Med* 2008;132:384-396.

8. Ring BZ, Seitz RS, Beck RA, et al. A novel five-antibody immunohistochemical test for subclassification of lung carcinoma. *Mod Pathol* 2009;22:1032-1043.

9. Kim DH, Kwon MS. Role of fine needle aspiration cytology, cell block preparation and CD63, P63 and CD56 immunostaining in classifying the specific tumor type of the lung. *Acta Cytol* 2010;54:55-59.

10. Vazquez MF, Koizumi JH, Henschke CI, et al. Reliability of cytologic diagnosis of early lung cancer. *Cancer* 2007;111:252-258.

11. Carvalho L. Reclassifying bronchial-pulmonary carcinoma: differentiating histological type in biopsies by immunohistochemistry. *Rev Port Pneumol* 2009;15:1101-1119.

12. Khayyata S, Yun S, Pasha T, et al. Value of P63 and CK5/6 in distinguishing squamous cell carcinoma from adenocarcinoma in lung fine-needle aspiration specimens. *Diagn Cytopathol* 2009;37:178-183.

13. Edwards SL, Roberts C, McKean ME, et al. Preoperative histological classification of primary lung cancer: accuracy of diagnosis and use of the non-small cell category. *J Clin Pathol* 2000;53:537-540.

14. Righi L, Graziano P, Fornari A, et al. Immunohistochemical subtyping of nonsmall cell lung cancer not otherwise specified in fine-needle aspiration cytology: a retrospective study of 103 cases with surgical correlation. *Cancer* 2011;117:3416-3423.

15. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205-216.

16. Therasse P, Eisenhauer EA, Verweij J. RECIST revisited: a review of validation studies on tumour assessment. *Eur J Cancer* 2006;42:1031-1039.

17. Pelosi G, Haspinger ER, Bimbatti M, et al. Does Immunohistochemistry Affect Response to Therapy and Survival of Inoperable Non-Small Cell Lung Carcinoma Patients? A Survey of 145 Stage III-IV Consecutive Cases. *Int J Surg Pathol* 2013.

18. Scagliotti GV, Vynnychenko I, Park K, et al. International, randomized, placebo-controlled, double-blind phase III study of motesanib plus carboplatin/paclitaxel in patients with advanced nonsquamous non-small-cell lung cancer: MONET1. *J Clin Oncol* 2012;30:2829-2836.

19. Lara PN, Jr., Douillard JY, Nakagawa K, et al. Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. *J Clin Oncol* 2011;29:2965-2971.

20. Travis WD, Brambilla E, Muller-Hermelink HK, et al. *Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press; 2004.

21. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. *J Natl Cancer Inst* 2005;97:339-346.

22. The Clinical Lung Cancer Genome Project (CLCGP) and Network Genomic Medicine (NGM). A genomics-based classification of human lung tumors. *Sci Transl Med* 2013;5: 209ra153. doi:10.1126/scitranslmed.3006802.

23. Travis WD, Brambilla E, Riely GJ. New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. *J Clin Oncol* 2013;31:992-1001.

24. Rossi G, Pelosi G, Barbareschi M, et al. Subtyping non-small cell lung cancer: relevant issues and operative recommendations for the best pathology practice. *Int J Surg Pathol* 2013;21:326-336.

25. Thunnissen E, Kerr KM, Herth FJ, et al. The challenge of NSCLC diagnosis and predictive analysis on small samples. Practical approach of a working group. *Lung Cancer* 2012;76:1-18.

26. Pelosi G, Fabbri A, Bianchi F, et al. DeltaNp63 (p40) and thyroid transcription factor-1 immunoreactivity on small biopsies or cellblocks for typing non-small cell lung cancer: a novel two-hit, sparing-material approach. *J Thorac Oncol* 2012;7:281-290.

27. Bishop JA, Teruya-Feldstein J, Westra WH, et al. p40 (DeltaNp63) is superior to p63 for the diagnosis of pulmonary squamous cell carcinoma. *Mod Pathol* 2012;25:405-415.

28. Pelosi G, Rossi G, Cavazza A, et al. DeltaNp63 (p40) distribution inside lung cancer: a driver biomarker approach to tumor characterization. *Int J Surg Pathol* 2013;21:229-239.

29. Loo PS, Thomas SC, Nicolson MC, et al. Subtyping of undifferentiated non-small cell carcinomas in bronchial biopsy specimens. *J Thorac Oncol* 2010;5:442-447.

30. Terry J, Leung S, Laskin J, et al. Optimal immunohistochemical markers for distinguishing lung adenocarcinomas from squamous cell carcinomas in small tumor samples. *Am J Surg Pathol* 2010;34:1805-1811.

31. Rossi G, Papotti M, Barbareschi M, et al. Morphology and a limited number of immunohistochemical markers may efficiently subtype non-small-cell lung cancer. *J Clin Oncol* 2009;27:e141-142; author reply e143-144.

32. Kadivar M, Boozari B. Applications and limitations of immunohistochemical expression of "Napsin-A" in distinguishing lung adenocarcinoma from adenocarcinomas of other organs. *Appl Immunohistochem Mol Morphol* 2013;21:191-195.

33. Masai K, Tsuta K, Kawago M, et al. Expression of squamous cell carcinoma markers and adenocarcinoma markers in primary pulmonary neuroendocrine carcinomas. *Appl Immunohistochem Mol Morphol* 2013;21:292-297.

34. Monica V, Ceppi P, Righi L, et al. Desmocollin-3: a new marker of squamous differentiation in undifferentiated large-cell carcinoma of the lung. *Mod Pathol* 2009;22:709-717.

35. Sigel CS, Moreira AL, Travis WD, et al. Subtyping of non-small cell lung carcinoma: a comparison of small biopsy and cytology specimens. *J Thorac Oncol* 2011;6:1849-1856.

36. Travis WD, Brambilla E, Noguchi M, et al. International association for the study of lung cancer/american thoracic society/european respiratory society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol* 2011;6:244-285.

FIGURE LEGENDS

Figure 1: Distribution graphs of diagnoses prior and after immunohistochemical investigation of NSCLC-NOS group. Abbreviations: ADC: adenocarcinoma; NSCLC-NOS: Non Small Cell Lung Cancer-Not Otherwise Specified; IHC: Immunohistochemistry; SQC: Squamous Carcinoma.

Figure 2: Distribution of best responses to first line treatment in the different NSCLC groups. Abbreviations: PD: Progressive Disease; PR: Partial Response; SD: Stable Disease; ADC: adenocarcinoma; NSCLC: Non Small Cell Lung Cancer; NOS: Not Otherwise Specified; morpho: morphological; (m+f): morfhological and favor.

Figure 3: Kaplan-Meier curves of progression free (PFS) and overall survival (OS) in the patient series of morphological ADC and NSCLC-NOS.

Abbreviations: ADC: adenocarcinoma; NSCLC: Non Small Cell Lung Cancer; NOS: Not Otherwise Specified; morpho: morphological.

Figure 4: Kaplan-Meier curves of progression free (PFS) and overall survival (OS) in the patient series after immunohistochemical subtyping: A) PFS and OS of the three separate groups; B) all ADC, morphologically and immunohistochemically (m+f) defined were grouped together and compared to the group of NSCLC null phenotype. ADC: adenocarcinoma; NSCLC: Non Small Cell Lung Cancer; NOS: Not Otherwise Specified.

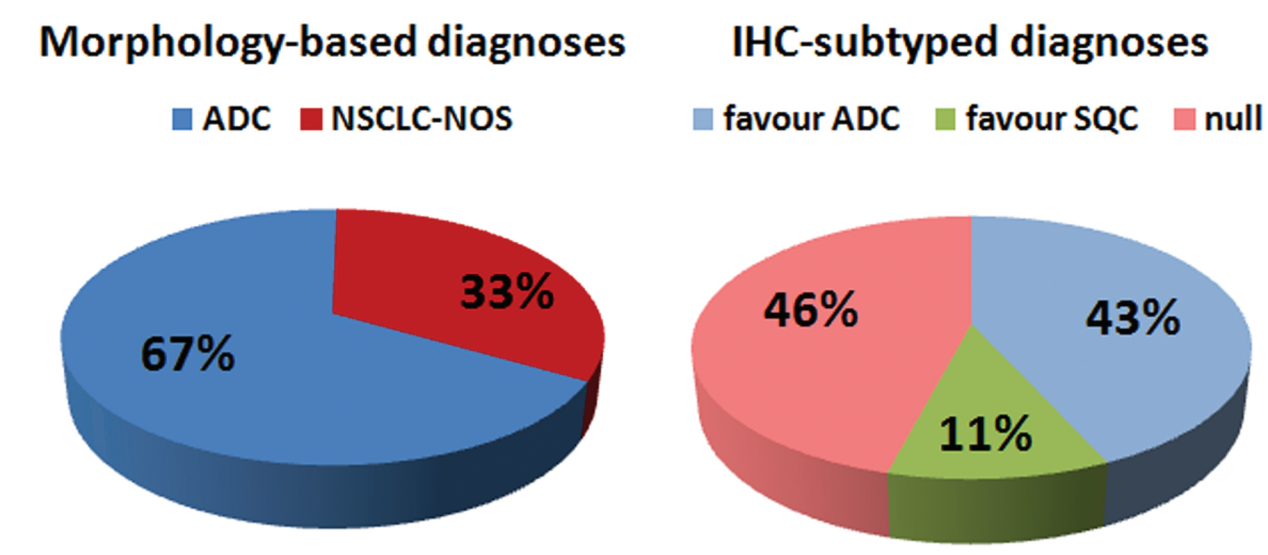
Table 1: Clinical-pathological characteristics of 224 patients.

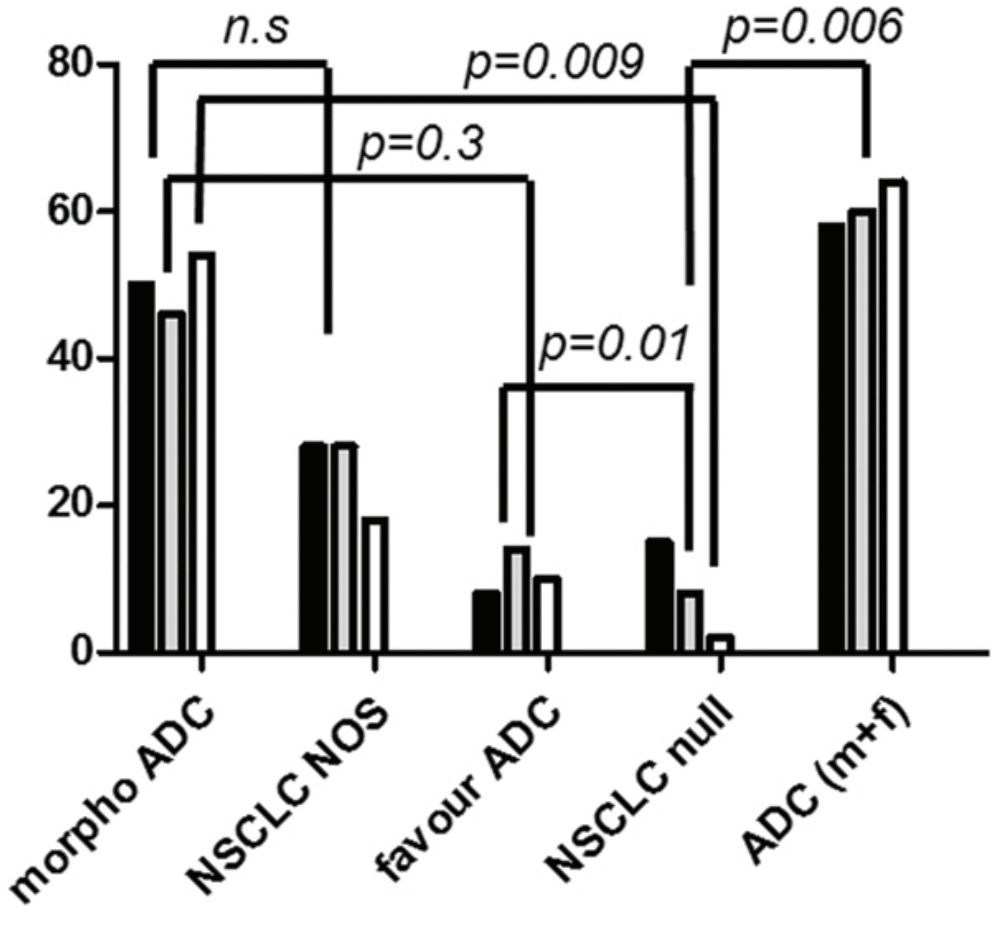
Patients characteristics	Overall #224 N (%)	morpho ADC #150 N (%)	IHC favour ADC #32 N (%)	NSCLC null #34 N (%)	IHC favour SQC #8 N (%)
Age, median (range)	62 (33-83)	62 (33-81)	65 (51-83)	61 (44-74)	67 (62-73)
Gender					
Males	156 (70)	102 (68)	21 (66)	25 (74)	8 (100)
Females	68 (30)	48 (32)	11 (34)	9 (26)	0
Stage					
IIIB	49 (22)	33 (22)	4 (13)	9 (26)	3 (37)
IV	175 (78)	117 (78)	28 (87)	25 (74)	5 (63)
Smoking					
Current	86 (38)	53 (35)	12 (38)	17 (50)	4 (50)
Former	100 (45)	68 (45)	13 (41)	15 (44)	4 (50)
No	38 (17)	29 (19)	7 (21)	2 (6)	0
PS					
0	160 (71)	110 (73)	19 (59)	25 (74)	6 (75)
1	58 (26)	36 (24)	12 (38)	9 (26)	1 (25)
2	6 (3)	4 (3)	1 (3)	0	1 (25)
Tissue sample					
Histological biopsies	94 (42)	72 (48)	10 (31)	8 (24)	4 (50)
Cytological blocks	130 (58)	78 (52)	22 (69)	26 (76)	4 (50)
Treatments					
Plat doublet+GEM	63 (28)	45 (30)	5 (16)	8 (23)	5 (63)
Plat doublet+TAX	30 (13)	19 (13)	3 (9)	5 (15)	3 (37)
Plat doublet+PEM	23 (10)	19 (13)	4 (13)	0	0
*Others**	20 (9)	10 (7)	7 (22)	3 (9)	0
*Clinical trials***	88 (39)	57 (38)	13 (41)	18 (53)	0
Best response					
CR	0 (0)	0	0	0	0
PR	73 (32.6)	46 (31)	14 (44)	12 (35)	2 (25)
SD	72 (32.1)	54 (36)	10 (31)	5 (15)	3 (37.5)
PD	78 (34.9)	50 (33)	8 (25)	17 (50)	3 (37.5)
RR (%)	33	31	44	35	25
DCR (%)	65	67	75	50	63
Survival					
AWD	11 (5)	10 (7)	1 (3)	0	0
DOD	213 (95)	140 (93)	31 (97)	34 (100)	8 (100)
Mean PFS (m)	6.8	7.3	7.1	3.8	7.4
Mean OS (m)	11.6	12.3	13.1	7.2	9.7

*Others include: monochemotherapy with GEM or PEM, carboplatin-paclitaxel-bevacizumab, cisplatin-etoposide.

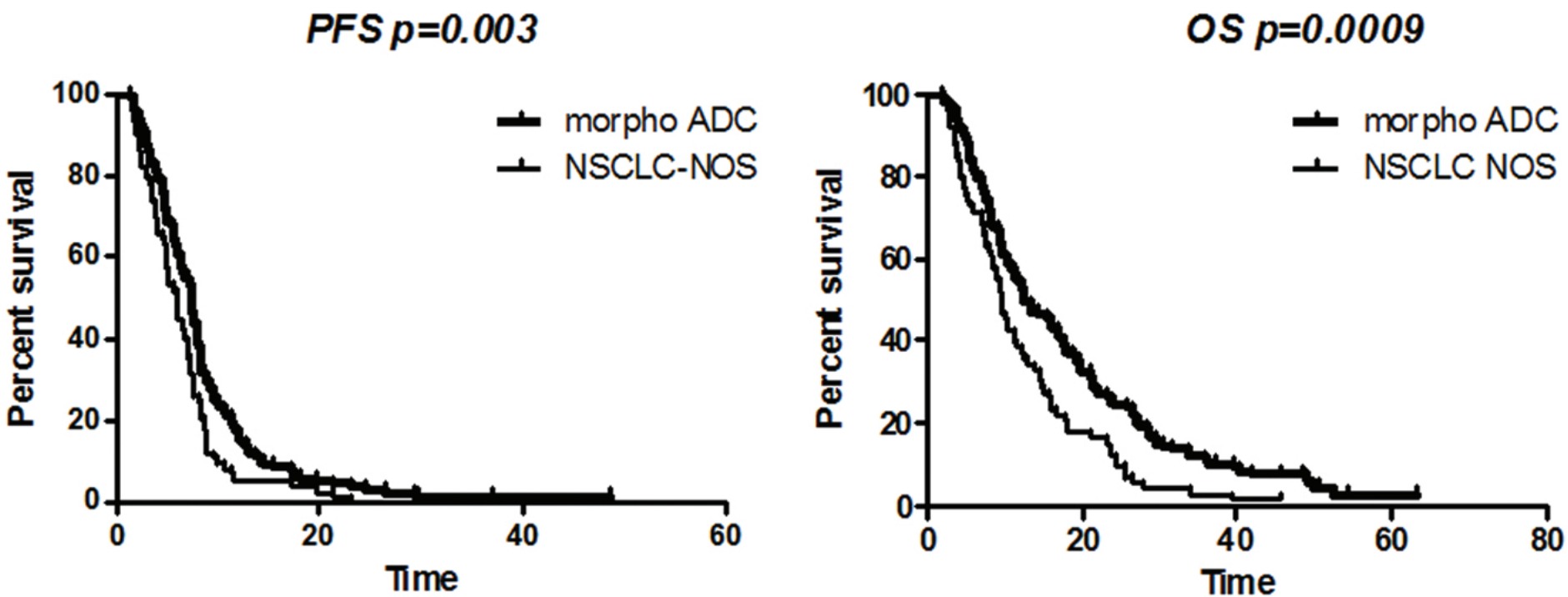
**I line treatments within clinical trials include: carboplatin-paclitaxel-AMG 706 (motesanib), carboplatin-paclitaxel-ASA 404 (vadimezan), carboplatin-paclitaxel-CP 751,871 (figitumumab), carboplatin-paclitaxel-sorafenib, cisplatin-gemcitabine-SAR240550 (iniparib), cisplatin-PEM-axitinib, gemcitabine-vandetanib, PEM-pazopanib, compassionate I line erlotinib or gefitinib.

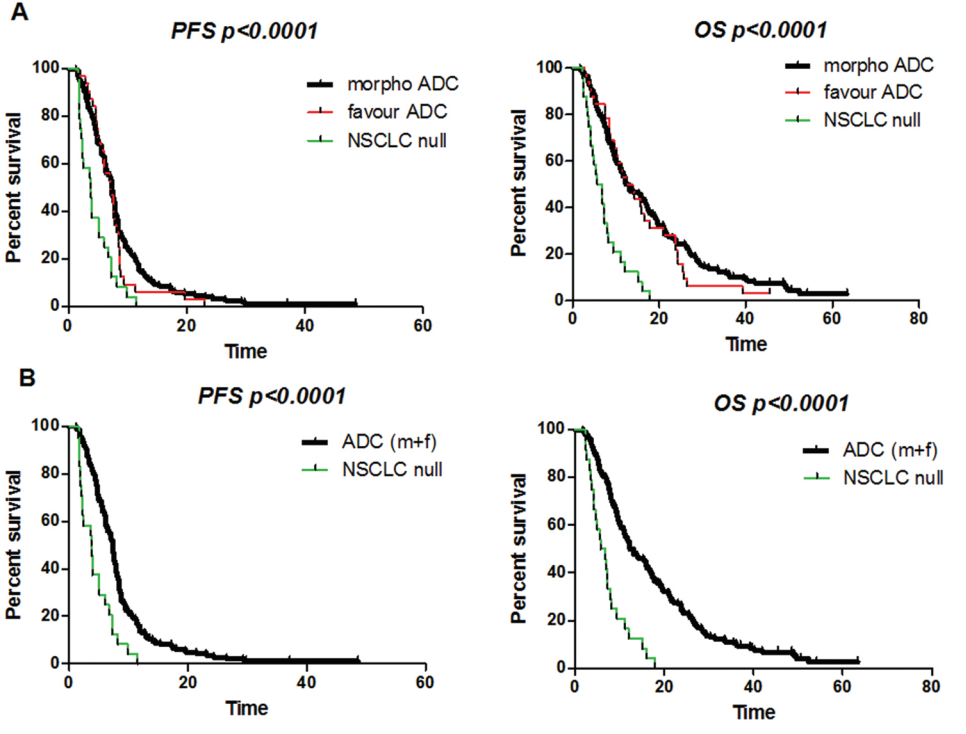
Abbreviations: ADC: adenocarcinoma; NSCLC-NOS: Non Small Cell Lung Cancer- Not Otherwise Specified; CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; AWD; Alive With Disease; DOD: Death Of Disease, GEM: Gemcitabine; TAX: Taxanes; PEM: Pemetrexed.


Morphology-based diagnoses
ADC
NSCLC-NOS
67%
33%
IHC-subtyped diagnoses
favour ADC
favour SQC
null
46%
43%
11%



	morpho ADC	NSCLC NOS	favour ADC	NSCLC null	ADC (m+f)
CR	0	0	0	0	0
PR	46	28	14	12	60
SD	54	18	10	5	64
PD	50	28	8	17	58
TOT	**150**	**74**	**32**	**34**	**182**
RR	31%	38%	44%	35%	33%
DCR	67%	62%	75%	50%	68%


PFS p=0.003
Percent survival
100
80
60
40
20
0
0
20
40
60
Time
morpho ADC
NSCLC-NOS
OS p=0.0009
Percent survival
100
80
60
40
20
0
0
20
40
60
80
Time
morpho ADC
NSCLC NOS


A
PFS p<0.0001
Percent survival
Time
morpho ADC
favour ADC
NSCLC null
OS p<0.0001
Percent survival
Time
morpho ADC
favour ADC
NSCLC null
B
PFS p<0.0001
Percent survival
Time
ADC (m+f)
NSCLC null
OS p<0.0001
Percent survival
Time
ADC (m+f)
NSCLC null